Identifying a Membrane Tripartite ATP-independent Periplasmic (TRAP) Transporter and a Transcriptional Regulator Gene in *Rhodobacter sphaeroides*

Undergraduate Research Thesis

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Abstract

This experiment relates to Rhodobacter sphaeroides, *a bacterium, and how it uses certain amino acids as its carbon source. Specific proteins and enzymes are required to uptake and convert glutamate and alanine into intermediates that the cell can use to make all of its constituents. To determine the identity of the genes involved in glutamate or alanine metabolism, mutant strains were created via random transposon mutagenesis and screened for a loss of the ability to use glutamate or alanine as a carbon source. Mutants Sp11-18-60 and Au11-38-158 showed positive growth with lactate and compromised growth on glutamate and aspartate; while mutant Sp11-10-236 did not grow with alanine as the carbon source. For both of the mutant strains, Sp11-18-60 and Au11-38-158, the same gene was interrupted which is annotated as coding for a subunit of a tripartite ATP-independent periplasmic (TRAP) membrane transporter. In* R. sphaeroides, *the specificity of this TRAP transporter, a membrane protein which moves compounds into the cell via a proton motive force, is unknown. This report provides data which support the idea that this TRAP transporter is specific for the uptake of glutamate and aspartate. It also shows that this TRAP transporter requires sodium, which cannot be replaced by potassium. The interrupted gene of Sp11-10-236 is annotated as coding for a transcriptional regulator. The target of this regulator protein is also unknown. To confirm the hypotheses that the TRAP transporter is involved in the uptake of glutamate and that the regulator is required for growth with alanine, non-mutated copies of the genes were introduced into the respective mutants via a plasmid, a process called complementation. Complementation of Au11-38-158 restored its growth on glutamate and aspartate, whereas complementation of Sp11-18-60 did not restore*

growth. Complementation of Sp11-10-236 has not been completed. Better understanding of bacteria's physiology facilitates genetic modification which may provide compounds that benefit humans.

Dedication

Dedicated to all the *R. sphaeroides* and *E. coli* cells that were poisoned, starved, lysed, mutated, burned alive, frozen, forced to copulate, and had their innards removed all in the name of science!

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Vita

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Introduction

Rhodobacter sphaeroides 2.4.1 is a rod-shaped, Gram-negative, purple bacterium found in ponds and lakes. Strain 2.4.1 has two sequenced chromosomes and five genomic plasmids (Suwanto & Kaplan, 1992). It is metabolically diverse, for example it can grow aerobically in the dark, anaerobically in the light, and anaerobically in the dark in the presence of dimethylsulfoxide (Mouncey *et al.*, 1997). It can also use many different carbon substrates and fix its own nitrogen. The carbon source can even come from amino acids, which will be the focus of this study. *R. sphaeroides* can also use amino acids as a nitrogen source, but this paper will not focus on that. To uptake these amino acids, different types of membrane transporters are used, which are laid out in Figure 1. Once the substrate is inside of the cell, specific enzymes convert

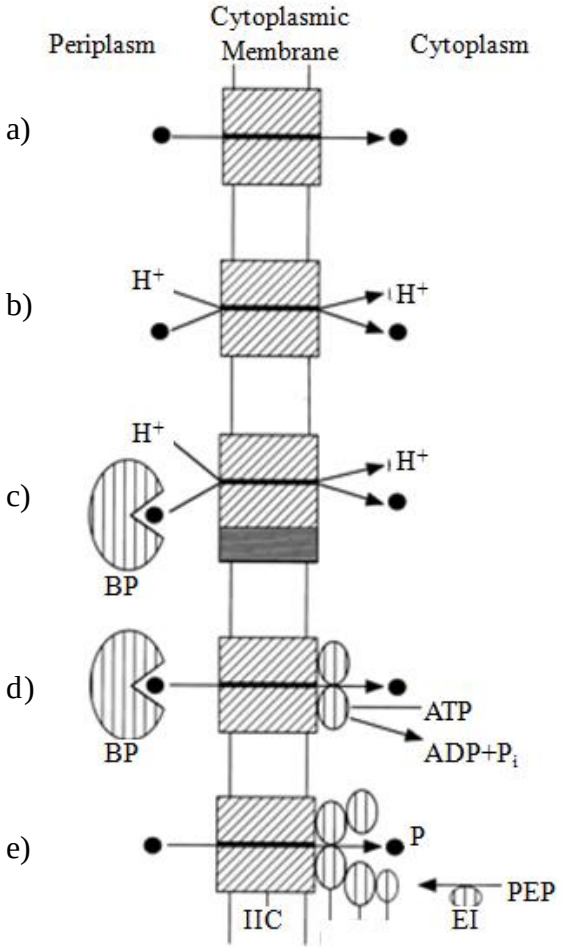


Figure 1: Five main types of membrane transporters. BP is a binding protein. a) facilitated diffusion b) secondary transport c) tripartite ATP-independent periplasmic (TRAP) transport d) ATP Binding Cassette (ABC) transport e) Phosphotransferase system. Taken from Rabus, *et al.* (1999).

the amino acid into a central carbon metabolic precursor. However, it is likely that these enzymes are not produced when the specific amino acid is not present, as this would waste energy. To minimize the amount of energy used, regulatory proteins are commonly produced to regulate the transcription of proteins that are unneeded in a particular environment.

It has been confirmed that *R. sphaeroides* contains an ABC transporter that uptakes glutamate (Jacobs *et al.*, 1995). The common ABC transporter requires the hydrolysis of ATP to provide energy to transport a molecule across the cell membrane, shown in Figure 1d. ABC transporters can either import or export a variety of molecules that vary in size, charge, or makeup (Rees *et al.*, 2009). Tripartite ATP-independent periplasmic (TRAP) transporters are similar to ABC transporters in that they use a periplasmic binding protein, but are different because they do not require ATP to transport molecules across the membrane, shown in Figure 1c. Instead they rely on a proton motive force. This force is an electrochemical gradient created by a more positive charge on the inner cell membrane compared to that of the cytoplasm. As the TRAP channel opens, an ion flows down its gradient while taking the substrate with it (Forward *et al.*, 1997). In this instance, the ion is likely Na^+ (Jacob *et al.*, 1996). Because the electrochemical gradient is only in one direction, TRAP transporters can only import molecules. The first TRAP transport system was identified in *Rhodobacter capsulatus* and was composed of three subunits DctP, DctQ, and DctM (Forward *et al.*, 1997). Prior to the classification of TRAP transporters, a study by Jacobs *et al.* (1996) showed that growth with glutamate could be restored for an ABC transport deficient *R. sphaeroides* strain by the addition of sodium. This was likely due to the presence of a glutamate specific TRAP transporter. Currently, the genomic sequence of the glutamate TRAP-T gene is unknown in *R. sphaeroides.*

Most enzymes have a regulatory protein, which influences transcription rates. Depending on the cell's requirements, the regulatory protein may bind to an operator, which may inhibit

transcription or increase transcription. A repressor inhibits transcription and works one of two ways: the protein may only bind to the operator if a specific ligand is present or the protein may release the operator in the presence of the ligand. On the other hand, activators, which increase transcription, only function by binding to the activation site in the presence of the ligand (Slonczewski & Foster, 2009). An example of a potential activator is the alanine dehydrogenase regulatory protein. The exact location on the genome of this regulator is not known in *R. sphaeroides* and will be further discussed in this paper.

This experiment attempted to characterize the genes necessary for the uptake or catabolism of amino acids glutamate and alanine. Three mutants were found to have compromised or negative growth on either glutamate or alanine. Two mutants with compromised glutamate growth may have an interrupted TRAP transporter gene. A mutant with negative growth on alanine may have an interrupted alanine dehydrogenase regulator gene.

Methods and Materials

Bacterial strains

The following bacterial strains were used in this experiment:

Rhodobacter sphaeroides 2.4.1 (DSMZ158), Sp11-18-60, Au11-38-158, and Sp11-10-236

Escherichia coli WM2672 (Larsen *et al.*, 2002), DH5α-λpir, DH5α, and SM10 (Simon *et al.*, 1983)

Media composition

Luria-Bertani (LB): 10 g tryptone, 5 g yeast extract, and 5 g sodium chloride per 1 liter. If the medium was used to make agar plates, 15 g Agar-Agar per 1 liter was added. The medium was autoclaved.

Minimal Medium (MM): 1.2 g NH_4Cl, 0.2 g $MgSO_4 \cdot 7\ H_2O$, 0.07 g $CaCl_2 \cdot 2\ H_2O$, and 10 mL trace element solution per 1 liter. The solution was made anaerobic with ten cycles, three minutes each, of degassing and gassing with nitrogen gas and was then autoclaved. After cooling, the following components were added: 1.5 mL vitamin solution, 15 mL 1 M phosphate buffer, and a carbon source of varying concentrations per 1 liter. If the medium was used to make agar plates, 18 g Agar-Agar per 1 liter was added.

Tris ethylenediaminetetraacetate(TE) buffer: 10 mM Tris-Cl (pH 8.0) and 1 mM EDTA

Vitamin solution: 100 mg cyanocobalamin, 300 mg pyridoxamine-2 HCl, 100 mg calcium-D(+)-pantothenate, 200 mg thiamindichlorid, 200 mg nicotinic acid, 80 mg 4-aminobenzoic acid, and 20 mg D(+)-biotin per 1 liter. The solution was sterilized using a 0.2 μm filter.

Phosphate buffer: 1 M K_2HPO_4 was titrated with 1 M KH_2PO_4 until the pH equaled 6.7. The solution was made anaerobic and autoclaved.

Trace element solution: 500 mg disodium ethylenediaminetetraacetate (EDTA), 300 mg $FeSO_4 \cdot 7 H_2O$, 3 mg $MnCl_2 \cdot 4 H_2O$, 5 mg $CoCl_2 \cdot 6 H_2O$, 1 mg $CuCl_2 \cdot 2 H_2O$, 2 mg $NiCl_2 \cdot 6 H_2O$, 3 mg $Na_2MoO_4 \cdot 2 H_2O$, 5 mg $ZnSO_4 \cdot 7 H_2O$, and 2 mg H_3BO_3 per 1 liter. Added sodium hydroxide until the pH equaled about 3. The solution was made anaerobic and autoclaved.

Antibiotics: For *R. sphaeroides*: 20 μg/mL kanamycin (kan), 25 μg/mL spectinomycin (spec). For *E. coli*: 50 μg/mL kanamycin (kan), 50 μg/mL spectinomycin (spec), 100 μg/mL ampicillin (amp). Antibiotics were sterilized using a 0.2 μm filter.

Growth conditions and storage

R. sphaeroides was grown at 30 ^oC either anaerobically in the light or aerobically in the dark. To make a stock culture, a single colony from an agar plate was inoculated in 5 mL LB medium with the appropriate antibiotic. The cells were pelleted using a centrifuge (1.5 min, 15,700 × g) and the supernatant was removed. The cells were resuspended in 1 mL LB+20% glycerol. To revive a *R. sphaeroides* culture from stock stored at -72 ^oC, a swab was streaked on an LB agar plate. The plate was incubated aerobically at 30 ^oC. An isolated colony was picked and inoculated into 5 mL LB media and grown aerobically in the dark. After a day, about 0.3 mL of liquid culture was transferred to 5 mL of defined minimal media and incubated anaerobically

at 30 ^{o}C. The cultures in minimal media were transferred to fresh media three times before an experiment was started. This eliminated carryover from the LB medium.

For growth experiments, *R. sphaeroides* was grown anaerobically in the light at 30 ^{o}C in 5 mL of minimal media plus 10 mM of a different sodium L- amino acid. Na- succinate or D/L Na- lactate was used as the control substrate. Amino acids, whose average carbon oxidation state was more negative than that of the cell carbon, required the addition of sodium bicarbonate. The concentration of $NaHCO_3$ was the same concentration as the carbon source. The initial growth experiment included amino acids alanine, arginine, asparagine, aspartate, cysteine, glutamate, glutamine, glycine, histidine, isoleucine, leucine, lysine, methionine, phenylalanine, proline, serine, threonine, tryptone, tyrosine and valine. Sodium succinate was used as the positive control. All cultures were grown anaerobically at 30 ^{o}C. The cell growth was quantified by measuring the optical density (OD) with a spectrometer, set to a wavelength of 578 nm. After the cultures had finished growing, the pH of the media was checked to be in the range of 6.8-7.4. The data were plotted on semi logarithmic graphs and the doubling and lag times were calculated.

Competent *E. coli* cells

Competent cells are those which are able to uptake a plasmid. To make competent *E. coli* DH5α-λpir, DH5α, and SM10 cells, an aliquot of a stock culture was streaked on an LB agar plate. A single colony was inoculated in 5 mL of LB medium and incubated overnight. From that overnight culture, 0.5-1.0 mL was inoculated in 50 mL of LB medium. When the $OD_{578\ nm}$ reached ~0.6, the 50 mL-flask was placed on ice for 10 minutes. The cells were pelleted in a centrifuge (5 min, 3,000 × g) and the supernatant was removed. The cells were resuspended in 2.7 mL of cold 0.1 M $CaCl_2$ and 2.3 mL of 50 % glycerol. The solution was divided into 250 µL aliquots and the tubes were dropped into liquid nitrogen and immediately stored at -72 ^{o}C.

Transformation

To transform competent *E. coli* to contain a plasmid, 200 μL of the competent cells were mixed with either 25 μL of a ligation reaction or 1 μL of an isolated plasmid. Another 200 μL of cells were mixed with 10 μL distilled H_2O as a control. All mixtures were incubated on ice for 20 minutes. The cells were then heat shocked at 42 ^oC for 2 minutes. Then the cells were incubated on ice for 10 minutes. A 1 mL volume of LB medium was added to the tube which was then incubated at 37 ^oC for 1 hour. In the case where a ligation plasmid was used, the cells were pelleted and most of the supernatant was removed. The cells were resuspended in the residual media and spread on LB agar plates containing appropriate antibiotics. For a blue/white screen, 45 μL of 2% isopropyl β-D-1-thiogalactopyranoside (IPTG) + 2% X-Gal in dimethylformamide was spread onto the plates. Only after the plates had dried were the cells spread on them. The cells were grown overnight at 37 ^oC. From these transformation plates, a single white colony was inoculated in 5 mL of LB+kan medium and grown at 37 ^oC. A white colony indicated that the *lac*Z gene on the plasmid had been interrupted from ligation with a DNA fragment.

Plasmid isolation

The Vince Schulz method was used to isolate the plasmid from cells. About 3 mL of LB media containing cells were pelleted and the supernatant was removed. The cells were resuspended in 100 μL of cold solution A, consisting of 50 mM glucose, 25 mM Tris-HCl, 10 mM EDTA (pH 7.8), and 200 μL of fresh solution B, consisting of 0.5 mL 2M NaOH, 4 mL H_2O, 0.5 mL 10% SDS. This reaction was incubated on ice for 5 min. Then, 150 μL 7.5 M ammonium acetate and 150 μL $CHCl_3$: isoamylalcohol were mixed into the solution. The cells were centrifuged (5 min, 15,700 × g) and 400 μL of the supernatant was added to 200 μL 30% PEG8000 +1.5 mM NaCl. The solution was kept on ice for 15 min and then centrifuged (10 min,

15,700 × g). The supernatant was completely removed. The remaining solution was resuspended in 30 µL TE buffer + 25 µg/mL RNAase. The Vince Schulz method was only used on plasmids that would not be sequenced. To isolate plasmids for sequencing, the more efficient Fermentas GeneJET Plasmid Miniprep kit was used.

Conjugation of *E. coli* and *R. sphaeroides*

Conjugation was used for complementation studies and transposon mutagenesis. It was performed by inoculating 3 mL of LB, containing the appropriate antibiotic, with a single colony of the recipient *R. sphaeroides* and another 3 mL LB, containing the appropriate antibiotic, with a single colony of the donor strain which contained the plasmid that would be transferred. The donor strain was *E. coli*-WM2672 for the random transposon mutagenesis and *E. coli*-SM10 for the complementation study. The cultures were grown overnight. A 500 µL aliquot of each of the overnight cultures were inoculated into 25 mL LB, with the appropriate antibiotic, and grown until all reached an $OD_{578\ nm}$ between 0.3 and 0.6. The cells were pelleted with a centrifuge (5 min, 3,000 × g) and the supernatant was removed. The cells were resuspended in 1 mL of LB medium. A one-to-one ratio of donor and recipient cells were mixed based on their OD, to a total of 1 mL. The cells were pelleted and the supernatant was removed. The cells were resuspended in 100 µL of LB medium. The 100 µL was dropped on an LB agar plate and incubated at 30 ^{o}C for ~ 24 hours. The *R. sphaeroides* cells were scraped from the drop of cells and resuspended in 100 µL of minimal media. For the complementation studies, the 100 µL was diluted 1:10, 1:100, and 1:1000 and spread on individual MM succinate agar plates, containing the appropriate antibiotics. The plates were incubated at 30 ^{o}C in the light for 5 days. From these plates an isolated colony was streaked on LB agar plates with the appropriate antibiotics and grown at 30 ^{o}C. After the cells had grown, a single colony was inoculated into 5 mL of LB medium with the

appropriate antibiotics, from which a stock culture was made and the plasmid was isolated. For the random transposon mutagenesis procedure, the cells in 100 μL of minimal media were spread on MM – D/L lactate + kan plates. The plates were incubated at 30 ^{o}C anaerobically in the light. Isolated colonies of *R. sphaeroides* were patched on MM agar plates containing kanamycin and either D/L-sodium lactate (positive control), L-alanine, L-asparagine, sodium L-glutamate, L-isoleucine, or L-valine as the carbon source. The plates were incubated aerobically at 30 ^{o}C. Figure 2 shows an example of the plates.

Identification of the transposon insertion site

To determine which gene had been interrupted by the transposon carrying a kanamycin resistant cassette, genomic DNA was isolated from the mutants with a Sigma-Aldrich GenElute Bacterial Genomic DNA Kit. To 26 μL of the genomic DNA 3 μL of 10 x reaction buffer and 1 μL *Nco*I (C/CATGG) was added. The reaction was incubated at 37 ^{o}C for 1 hour. The *Nco*I enzyme was inactivated by incubating the reaction at 65 ^{o}C for 20 minutes. To circularize the DNA fragments and create a plasmid, 29 μL ddH_2O, 5 μL 10 x ligase buffer, 15 μL *Nco*I digest, and 1.5 μL Fermentas T4 DNA ligase were mixed in a tube and incubated overnight at room temperature. The ligation plasmid was replicated by transforming it into *E. coli* DH5α-λpir. After the transformed culture had grown, the plasmid was isolated and primers tpnRL17-1 (5'-AAC AAG CCA GGG ATG TAA CG-3') and tpnRL13-2 (5'-CAG CAA CAC CTT CTT CAC GA -3') were used to sequence the flanking genomic regions of the transposon.

Digesting DNA

A typical digest contained 42 μL of the plasmid/DNA fragment, 5 μL of reaction buffer (10x), and 3 μL of restriction enzyme. The reaction was incubated at 37 ^{o}C for 5-8 hours. The

enzymes and buffers were removed from the digests with a QIAquick PCR purification kit. For a smaller scale digest, a master mix was made. This master mix contained 35 μL H_2O, 5 μL of reaction buffer (10x), and 2 μL of restriction enzyme. An aliquot of 3 μL was placed into separate tubes, followed by 7 μL of the DNA.

Creating a plasmid for complementation

The coding region for RSP_1412 and RSP_1413 plus an additional 244 base pairs upstream of the sequence was amplified via PCR. The reaction contained 69 μL of ddH_2O, 10 μL of pfu buffer (10x), 5 μL of TRAPcomp_for4 (TTG GAA TTC GCT CAG AAG GCG GGC TTG), 5 μL of TRAPcomp_rev6 (TAC GAT CTA GAG ATT GTC GAG CAG CCA GAC C), 2 μL of 100 mM dNTP, 7 μL of 500 μg/mL wt chromosomal DNA, and 1 μL of pfu polymerase. The program was set for 25 cycles at 97 ^oC for 5.75 min, then 65 ^oC for 30 sec, and then 72 ^oC for 6 min. The final cycle was at 65 ^oC for 5 min and 72 ^oC for 15 min. The final product was called resTRAP. The 4 kb product was run on an electrophoresis gel and the DNA was extracted from the gel using a Fermentas GeneJET Gel Extraction Kit. The resTRAP PCR product and a high replicating vector plasmid, pUC19, were cut with restriction enzymes *Eco*RI (GAATTC) and *Xba*I (TCTAGA). ResTRAP was then ligated with pUC19 to create a new plasmid pJAA01.

About 3 μL of plasmid pJAA01was digested with *Bam*HI. The gel showed DNA band sizes of 5.4 kb, 3.1 kb, and 0.3 kb. The process, starting with the transformation, was performed again. Plasmids with the correct band size were submitted for DNA sequencing. The primers used to sequence pJAA01 were pUC Forward (CGC CAG GGT TTT CCC AGT CAC GAC), pUC Reverse (CCT GTG TGA AAT TGT TAT CCG CT), SecTRAPF1 (ATC GAA GGC CTG GGC AAG CTC), SecTRAPR1 (ATT CCG GGC GGC ATG TAT C), and SecTRAPF3 (AGC ACG GCC ATC ACG ATC CAG TC). The sequencing showed that no mutations occurred to

the DNA during the PCR process. Once the sequence of the plasmids was confirmed, the genes were ligated to pBBR-sm2-MCS5 (Kovach *et al.*, 1995). Plasmids pJAA01 and pBBR were digested with *Xba*I and *Eco*RI. The sticky ends of pJAA01and pBBR were ligated to create a new plasmid pJAA012. Figure 3 shows the PCR product and a schematic of the cloning strategy.

The coding region for RSP_0722 plus an additional 240 base pairs upstream of the sequence was amplified via PCR. The RSP_0722 reaction contained 69 μL of ddH_2O, 10 μL of pfu buffer (10x), 5 μL of RSP0722comp_for1 (TTC ATG CGG ATC CAG CGC CGC TGA TCC TTC), 5 μL of RSP0722comp_rev1 (AGT ATC TAG AAG CAC CTC GTG CCC GTG ACT TG), 2 μL of 100 mM dNTP, 7 μL of 500 μg/mL wt chromosomal DNA, and 1 μL of pfu polymerase. The program was set for 40 cycles at 97 ^{o}C for 5.75 min, then 61 ^{o}C for 30 sec, and then 72 ^{o}C for 1 min. The final cycle was at 61 ^{o}C for 5 min and 72 ^{o}C for 15 min. The final product was called Sp10. The 0.7 kb product was run on an electrophoresis gel and the DNA was extracted from the gel using a Fermentas GeneJET Gel Extraction Kit. The Sp10 PCR product and a high replicating vector plasmid, pUC19, were cut with restriction enzymes *Bam*HI (GGATCC) and *Xba*I. Sp10 was then ligated with pUC19 to create a new plasmid pJAA02.

About 3 μL of plasmid pJAA02 was digested with *Pst*I (CTGCAG). The gel showed DNA band sizes of 5.1 kb, 0.3 kb, and 0.18 kb. The process, starting with the transformation, was performed again. Plasmids with the correct band size were submitted for DNA sequencing. The primers used to sequence pJAA02 were pUC Forward and pUC Reverse. Plasmids pJAA02 and pBBR were digested with *Bam*HI and *Xba*I. The sticky ends of pJAA02 and pBBR were ligated to create a new plasmid pJAA022. Figure 4 shows the PCR product and a schematic of the cloning strategy.

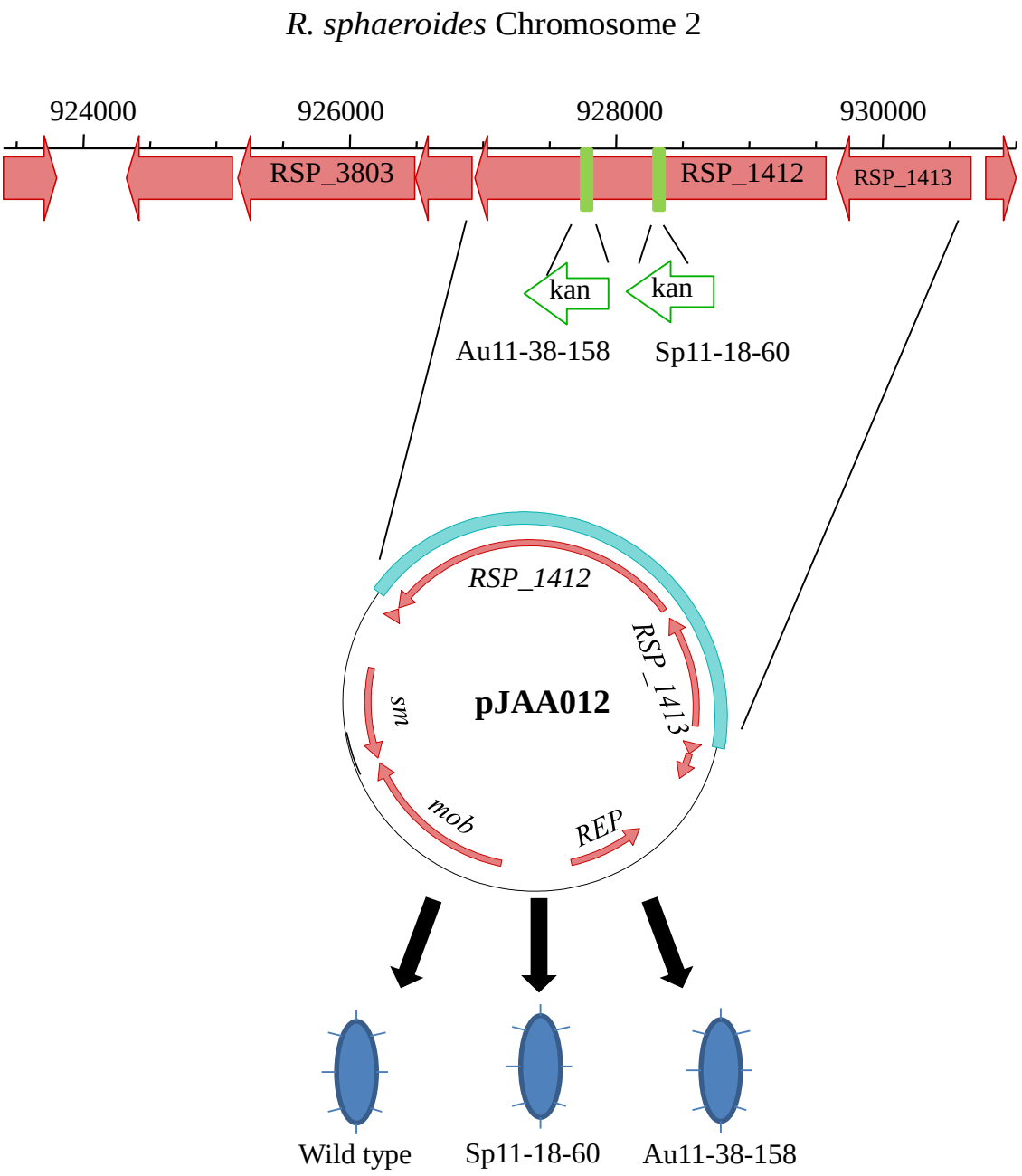


Figure 3 : The wild type genomic region was amplified via PCR and inserted into the pBBRsm2MCS5 vector plasmid, creating pJAA012. Plasmid pJAA012 was transformed into *R. sphaeroides* wild type and mutants Au11-38-158 (kan insertion site: 927776-927767 bp) and Sp11-18-60 (kan insertion site: 928119-928111) for complementation of the mutated genes. *Mob* and *REP* are required for plasmid replication in *R. sphaeroides*. *Sm* is a spectinomycin resistance gene.

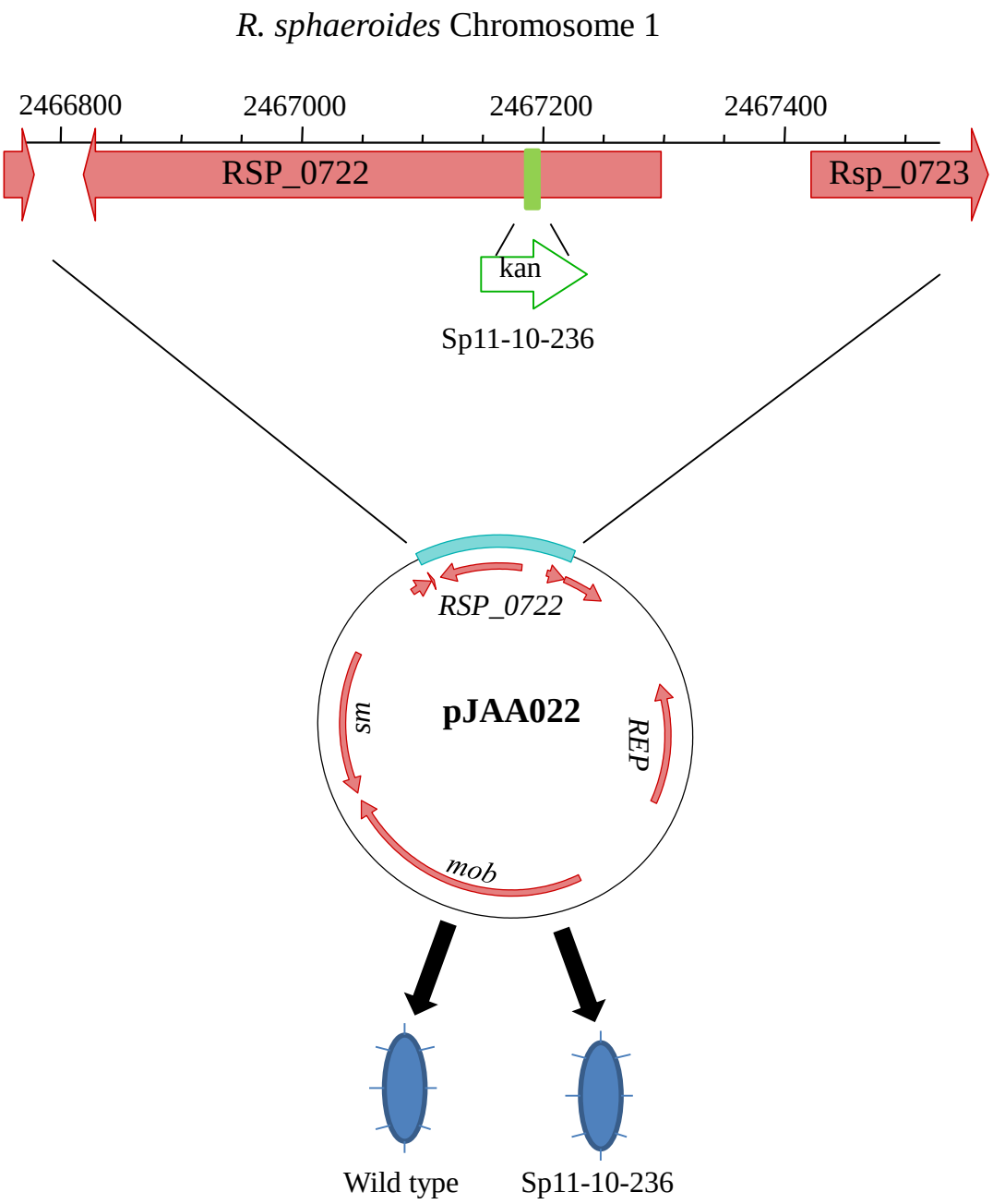


Figure 4 : The wild type genomic region was amplified via PCR and inserted into the pBBRsm2MCS5 vector plasmid, creating pJAA022. Plasmid pJAA022 was transformed into *R. sphaeroides* wild type and mutant Sp11-10-236 (kan insertion site: 2467200-2467190 bp) for complementation of the mutated gene. RSP_0723 likely encodes for alanine dehydrogenase. *Mob* and *REP* are required for plasmid replication in *R. sphaeroides*. *Sm* is a spectinomycin resistance gene.

Results

Use of amino acids as the sole carbon source by *Rhodobacter sphaeroides*

In order to determine which L-amino acids *R. sphaeroides* could use as a sole carbon source, cultures were grown anaerobically in the light in liquid minimal media containing one of the common 20 L-amino acids. Amino acids whose average carbon oxidation state was more negative than that of the cell carbon required the addition of sodium bicarbonate. Table 1 shows the doubling times for the different amino acids where growth was observed. Growth with serine or threonine was only tested once; therefore, the standard deviation could not be calculated. For other amino acids, the doubling time from multiple experiments varies due to slight differences in the growth conditions. No growth was observed on glutamine and serine until after several hours. *R. sphaeroides* is observed to use over half of

Table 1: Average growth parameters ± the standard deviation (number of experiments) for cultures grown in defined media containing 10 mM of different L - amino acids. Cultures were grown anaerobically in the light at 30 ^{o}C.

Carbon Source	$NaHCO_3$ concentration (mM)	Average Doubling Time (hr)
Alanine	0	10 ± 2 (4)
Asparagine	0	17 ± 1 (2)
Na^+ Aspartate	0	17 ± 7 (3)
Na^+ Glutamate	0	8 ± 1 (4)
Glutamine †	0	12 ± 2 (5)
Glycine	0	††
Histidine	0	49 ± 19 (2)
Isoleucine	10	10 ± 1 (5)
Lysine	10	46 ± 6 (2)
Proline	10	8 ± 0 (2)
Serine †	0	15 (1)
Threonine	0	34 (1)
Valine	10	††

* *R. sphaeroides* did not grow on arginine, cysteine, leucine/$NaHCO_3$, methionine/$NaHCO_3$, phenylalanine/$NaHCO_3$, tryptophan/$NaHCO_3$, and tyrosine/$NaHCO_3$ as the carbon source.

† Glutamine had a lag time of 64 ± 19 (5) hr. Serine had a lag time of 15 hr.
†† Glycine and valine did not show exponential growth.

the naturally occurring amino acids as the sole carbon source but is unable to grow with arginine, cysteine, leucine, methionine, phenylalanine, tryptophan and tyrosine under the given growth conditions.

Random transposon mutagenesis

In order to determine which genes were required for growth with amino acids, a different gene per cell was rendered nonfunctional by random transposon mutagenesis. Based on the fast doubling time of the cells, alanine, asparagine, glutamate, isoleucine, and valine were chosen as substrates for the random transposon mutagenesis experiment. To create the different mutants, a suicide plasmid carrying a transposon, containing a kanamycin resistant cassette, was introduced into *R. sphaeroides*. The transposon randomly inserted itself into the genome. Students of a microbial genetics class screened the random mutants on the aerobic plates for a negative growth phenotype with the different amino acids, with lactate as the positive control. Figure 2 shows an example of the screening plates. Once isolated, the growth phenotypes of the mutants were confirmed by growing the strains anaerobically in the light in liquid minimal media containing the respective amino acid. Table 2 summarizes the data for all of the characterized mutants created via random transposon mutagenesis. Mutants Sp11-18-60, Au11-38-158, and Sp11-10-236 have an interesting phenotype and were followed up with a complementation experiment.

Table 2: Summary of characterized *R. sphaeroides* transposon mutants isolated during a microbial genetics class in 2011

Mutant Name	RSP_	Chromosome/ Plasmid	Region of Insertion	Direction of Transposon Relative to Gene (Same/Opposite)	Effected Protein*	Photoheterotrophic Growth Phenotype			
						No Growth	Longer Doubling Time	Long Lag	Low Growth Yield
Sp11-18-162-1D	0398	1	2130080-2130071	S	Glu/Leu/Phe/Val dehydrogenase	Gln	Glu	Val	
Sp11-9-215	0398	1	2130334-2130320	S	Glu/Leu/Phe/Val dehydrogenase	Gln	Glu	Val	
Sp11-32-212**	1471	1	56544-56536	O	Hypothetical protein	Gln			Val
Sp11-10-236	0722	1	2467200-2467190	O	Regulator	Ala			
Au11-38-158	1412	2	927776-927767	S	TRAP-T family transporter with fused DctQ/DctM subunits		Glu	Gln	
Sp11-18-60	1412	2	928119-928111	S	TRAP-T family transporter with fused DctQ/DctM subunits		Glu	Gln	
Sp11-19-122	3981	P-B	71919-71910	O	Conserved hypothetical protein		Asn	Val, Glu	
Sp11-JAA-510	IG	1	624105-624097	NA	Intergenic Region		Ile	Ile, Val	
Sp11-9-88	2047	1	642915-642923	S	Hypothetical protein		Ile		Ile
Sp11-15-74	0612	1	2351371-2351369	S	RhtB family transporter/ threonine efflux		Ile		
Sp11-29-160-1	2643	1	1286018-1286028	S	Glutamate-ammonia-ligase adenylyltransferase	Val, Gln	Ile	Glu	Ile
Sp11-JAA-286	2207	1	820887-820876	S	Purine nucleoside phosphorylase	Gln	Lac, Asn	Glu	
Sp11-KS-40	0160	1	1876563-1876555	O	UPD-glucuronate decarboxylase/epimerase	Val	Lac, Ile, Glu	Gln	
Sp11-34-64	3669	2	787404-787413	S	Conserved hypothetical protein				Lac, Ile, Val, Asn

P-B= Plasmid B IG= Intergenic region NA= Not applicable

* The annotation was taken from the NCBI website

** Cells clumped together. Growth was determined by relative amounts of clumps

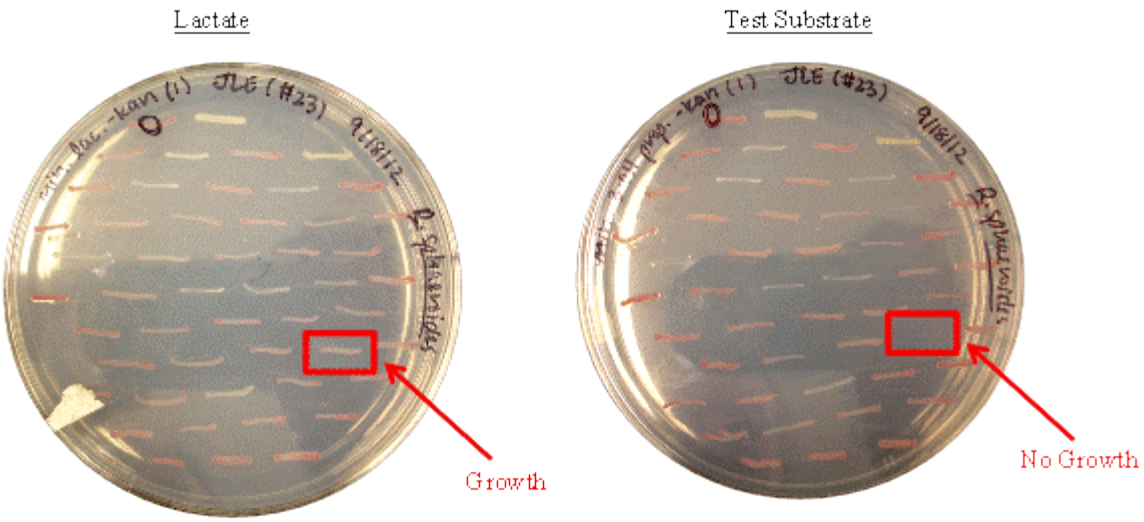
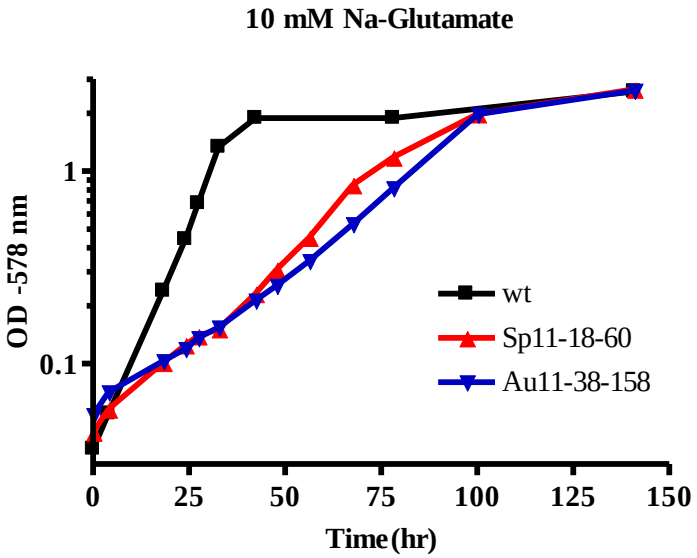


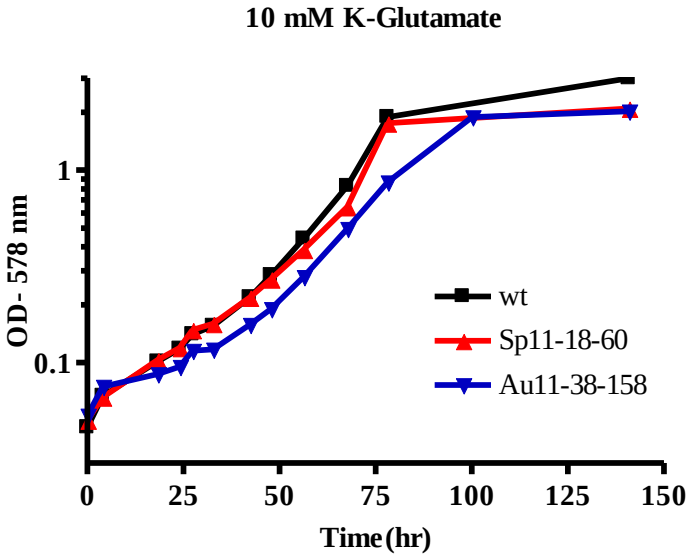
Figure 2: An example of minimal media agar plates, containing either lactate (positive control) or the test substrate. Individual *R. sphaeroides* cultures were streaked on both plates and grown at 30 °C in the dark. No growth indicated that a mutation had occurred in a gene which was required for growth on that substrate.

The glutamate mutants

Mutants Sp11-18-60 and Au11-38-158 have an insertion in the same gene that possibly codes for DctQ/DctM subunits of a TRAP transporter. Figure 5 shows the growth of mutants Sp11-18-60 and Au11-38-158 in liquid minimal media containing glutamate as the sole carbon source. These mutants show slowed growth with sodium glutamate as the carbon source; the mutants' doubling time is more than twice as long as that of the wild type.



Strain	Doubling Time (hr)
wt	6.9
Sp11-18-60	16.5
Au11-38-158	15.5



Strain	Doubling Time (hr)
wt	14
Sp11-18-60	17
Au11-38-158	14

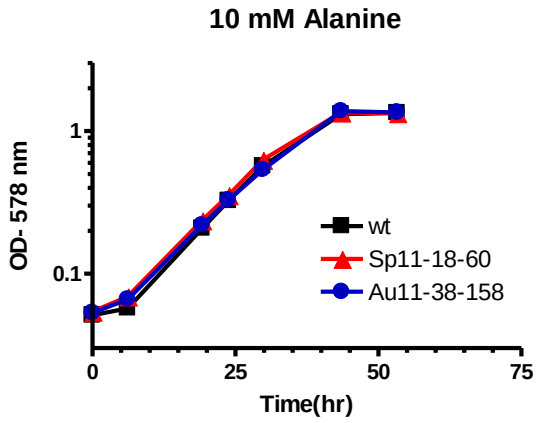
Figure 5 : *R. sphaeroides* wild type and mutant strains Sp11-18-60 and Au11-38-158 grown in minimal media containing 10 mM sodium glutamate and 10 mM potassium glutamate. The cultures were incubated anaerobically in the light at 30 ^{o}C

Sodium versus potassium glutamate

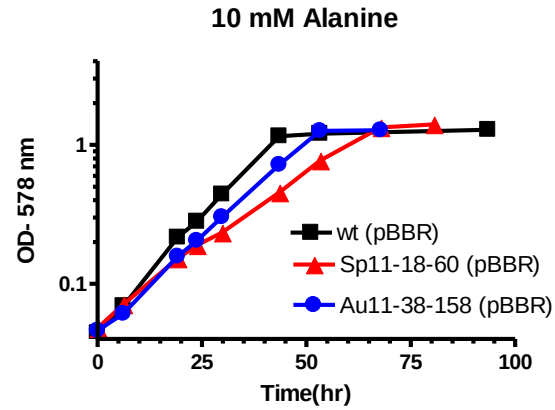
Strains Au11-38-158, Sp11-18-60, and the wild type were grown in liquid minimal media containing either sodium glutamate or potassium glutamate as the carbon source. Figure 5 shows the growth curves of the three strains for sodium glutamate and potassium glutamate. In previous experiments, Au11-38-158 and Sp11-18-60 had a longer doubling time than the wild type when grown with sodium glutamate. However, when grown with potassium glutamate the doubling time of the mutant strains was the same as the wild type's. It is also important to note that the doubling time of wild type in potassium glutamate was similar to the doubling times of the mutants when grown in sodium glutamate.

Complementation of the TRAP gene

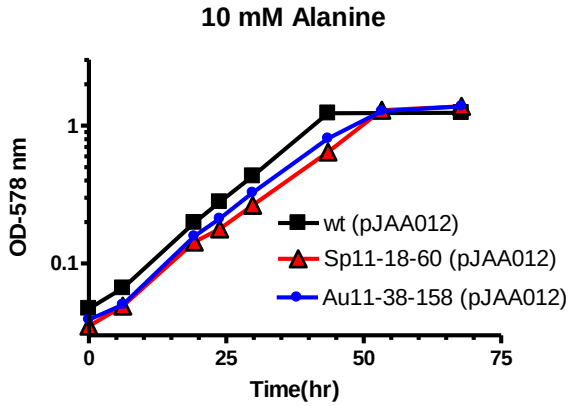
Strains Au11-38-158, Sp11-18-60, and the wild type were grown in liquid minimal media containing 10 mM of either alanine (positive control), sodium glutamate, or sodium aspartate. Figures 6, 7, and 8 show the growth curves for this experiment. All of the strains have similar growth with alanine, Figure 6. The mutants and the mutants with the pBBR vector show compromised growth with glutamate compared to wild type and the wild type containing the pBBR vector, Figure 7. The Au11-38-158 (pJAA012) strain has restored growth with glutamate, whereas Sp11-18-60 (pJAA012) does not. The mutants and the mutants containing the pBBR vector also have compromised growth with aspartate, Figure 8. The Au11-38-158 (pJAA012) strain has restored growth with aspartate, whereas Sp11-18-60 (pJAA012) does not.



Strain	Doubling Time (hr)
wt	7.3
Sp11-18-60	7.7
Au11-38-158	7.3

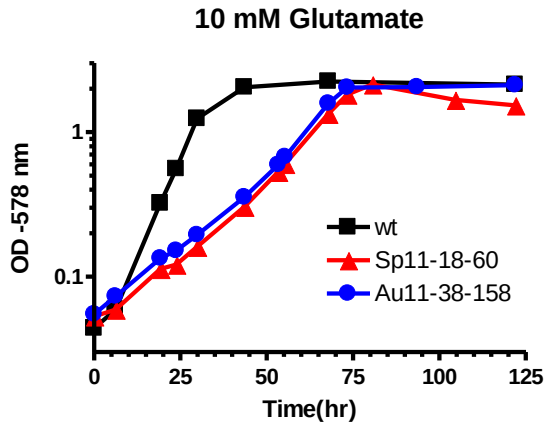


Strain	Doubling Time (hr)
wt (pBBR)	9.0
Sp11-18-60 (pBBR)	13.3
Au11-38-158 (pBBR)	11.0

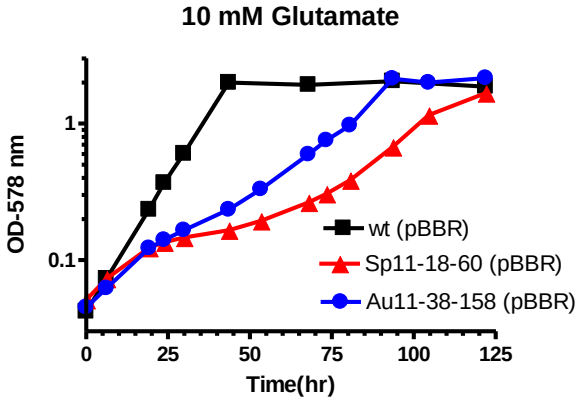


Strain	Doubling Time (hr)
wt (pJAA012)	9.3
Sp11-18-60 (pJAA012)	10.0
Au11-38-158 (pJAA012)	10.3

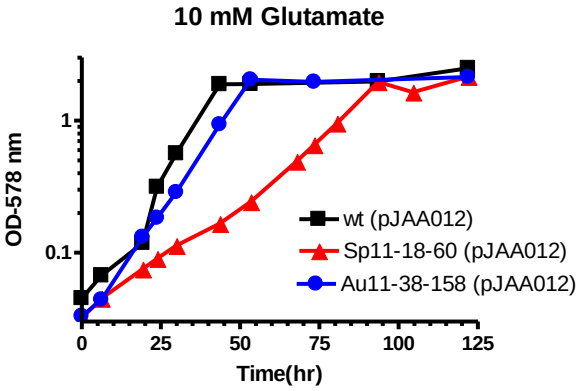
Figure 6 : ***R. sphaeroides*** **wild type and mutants Sp11-18-60 and Au11-38-158, with or without plasmid pBBR or pJAA012, grown in minimal media containing 10 mM alanine as the positive control. The cultures were incubated anaerobically in the light at 30 °C.**



Strain	Doubling Time (hr)
wt	5.2
Sp11-18-60	10.5
Au11-38-158	12.5

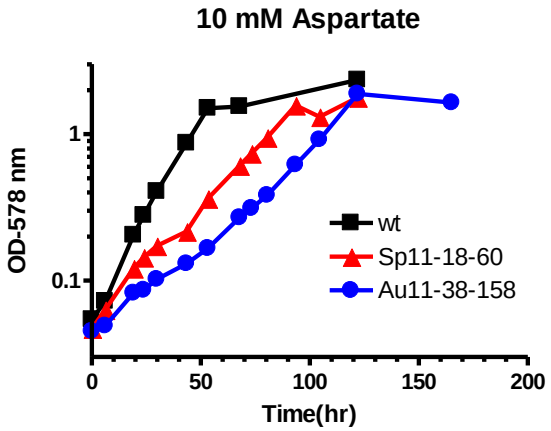


Strain	Doubling Time (hr)
wt (pBBR)	7.8
Sp11-18-60 (pBBR)	14.5
Au11-38-158 (pBBR)	17.0

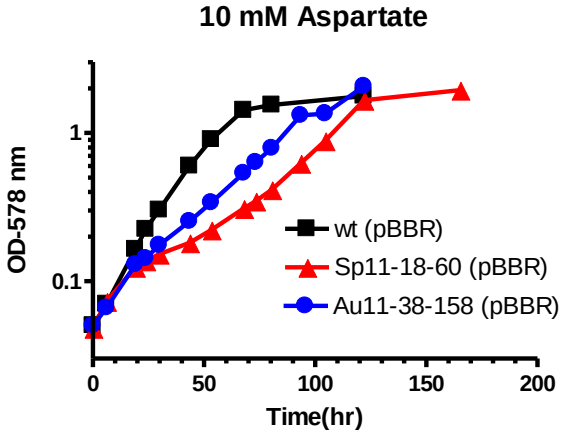


Strain	Doubling Time (hr)
wt (pJAA012)	7.3
Sp11-18-60 (pJAA012)	14.0
Au11-38-158 (pJAA012)	8.3

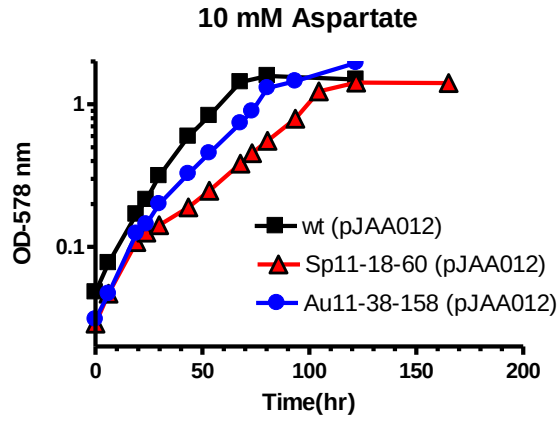
Figure 7 : *R. sphaeroides* wild type and mutants Sp11-18-60 and Au11-38-158, with or without plasmid pBBR or pJAA012, grown in minimal media containing 10 mM sodium glutamate. The cultures were incubated anaerobically in the light at 30 °C.



Strain	Doubling Time (hr)
wt	15.0
Sp11-18-60	23.7
Au11-38-158	24.5



Strain	Doubling Time (hr)
wt (pBBR)	15.5
Sp11-18-60 (pBBR)	21.5
Au11-38-158 (pBBR)	22.3



Strain	Doubling Time (hr)
wt (pJAA012)	17.0
Sp11-18-60 (pJAA012)	24.5
Au11-38-158 (pJAA012)	19.5

Figure 8 : ***R. sphaeroides*** **wild type and mutants Sp11-18-60 and Au11-38-158, with or without plasmid pBBR or pJAA012, grown in minimal media containing 10 mM sodium aspartate. The cultures were incubated anaerobically in the light at 30 ^{o}C.**

The alanine mutant

Mutant Sp11-10-236 has an insertion in a gene that possibly codes for a regulatory protein, which causes inhibited growth when grown in liquid media containing alanine. Figure 9 shows the growth of mutant Sp11-10-236 in liquid minimal media containing alanine as the sole carbon source.

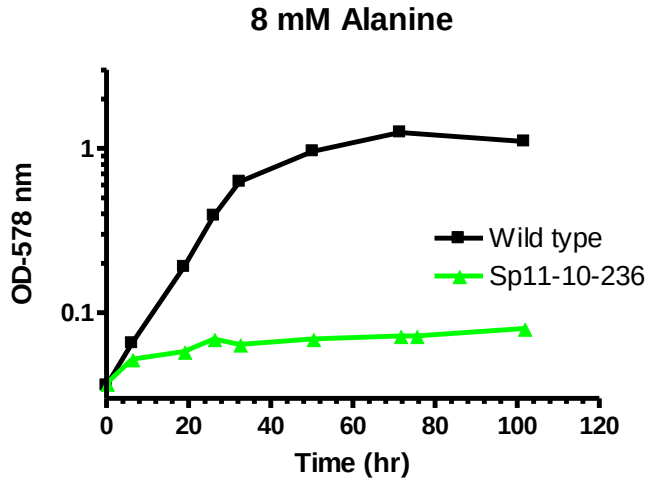


Figure 9 : ***R. sphaeroides*** **wild type and mutant strain Sp11-10-236 grown in minimal media containing 8 mM alanine. The cultures were incubated anaerobically in the light at 30 ^{o}C.**

Discussion

R. sphaeroides has the ability to use several amino acids as the sole carbon source when grown anaerobically in the dark at 30 ^{o}C in minimal media. It is possible that *R. sphaeroides* could not grow on arginine, cysteine, leucine/ $NaHCO_3$, methionine/ $NaHCO_3$, phenylalanine/ $NaHCO_3$, tryptophan/ $NaHCO_3$, and tyrosine/ $NaHCO_3$ because of an unknown missing nutrient in the media. The doubling times and lag times varied for each amino acid, indicating that the cell uses different metabolic processes for each. In fact, the mechanisms are specific enough that in most cases there is little correlation of growth with amino acids with similar structural or chemical properties.

Other large influences on the growth rate are the type and amount of transporters. Mutants Sp11-18-60 and Au11-38-158 have a transposon which randomly inserted itself into a gene annotated as a TRAP transporter. This transporter is specific for glutamate and aspartate based on the slow growth with sodium glutamate and aspartate as the carbon source. Growth is still possible because *R. sphaeroides* has an ABC transporter that uptakes glutamate and aspartate (Jacobs *et al.,* 1995). Therefore, it is reasonable to suggest that even in the mutants this ABC transporter is still functioning but the lack of the TRAP transporter means that import of glutamate or aspartate is slower, leading to slower growth with these compounds. Complementing the TRAP gene in Au11-38-158 restored the growth with glutamate and aspartate to wild type levels; confirming that gene RSP_1412 is involved in glutamate and aspartate transport. Based on Jacobs' (1996) study, this transporter has a higher affinity for glutamate. The growth of Sp11-18-60 with glutamate and aspartate was not restored by the insertion of

pJAA012 (a plasmid containing the functional TRAP gene). This may have to do with the location of the transposon, which still allows transcription of a membrane protein that is unable to transport. Presence of this mutant protein in Sp11-18-60 (pJAA012) would interfere with transport by the functional TRAP protein. The next step would be to make a clean deletion of the TRAP gene so that no transcription could occur.

When these mutants were grown with potassium glutamate, they appeared to have no growth defect compared to the growth of wild type with potassium glutamate. However, the doubling time of wild type when grown with sodium glutamate is shorter than the doubling time with potassium glutamate. In fact, when grown with potassium glutamate, the growth rate of wild type is just as slow as the growth rate of the mutants when grown with sodium glutamate. This means that this transporter requires the presence of sodium to function, which is further evidence that the mutated gene codes for a TRAP transporter.

Mutant Sp11-10-236 has a mutation in a gene annotated to encode a regulator, which is directly adjacent to the alanine dehydrogenase gene. While the wild type is able to grow on alanine, Sp11-10-236 is not, indicating that the gene RSP_0722 is required for growth with alanine. Alanine dehydrogenase is the only enzyme required to convert alanine into a central carbon metabolite, as seen in Figure 10. This and the location of the regulatory gene next to the gene coding for alanine dehydrogenase suggests that this regulator gene codes for an activator of the alanine dehydrogenase gene. The complementation experiment for this is in progress.

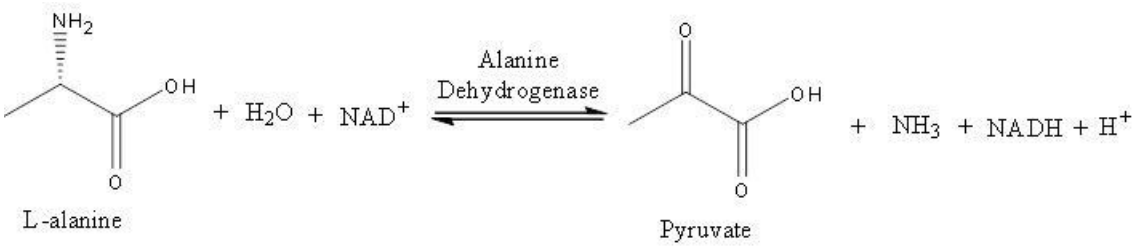


Figure 10 : **Alanine is converted to pyruvate by the enzyme alanine dehydrogenase**

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